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o-RAFFINOSE CROSSLINKING SUBSTANTIALLY AMELIORATES THE VASOCONSTRICTIVE AND NITRIC-OXIDE-INACTIVATING EFFECTS OF UNMODIFIED HUMAN HEMOGLOBIN IN THE RAT

BY

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ABSTRACT

We studied the hemodynamic effects of a 20% exchange transfusion with whole blood or highly purified human hemoglobin Ao. We compared the effects of unmodified hemoglobin with o-raffinose crosslinked oligomers of hemoglobin (modified hemoglobin). The unmodified SFH increased systemic vascular resistance (SVR) by ~40%, and renal vascular resistance (RVR) by ~25%. Cardiac output was markedly reduced (by ~20%) and mean arterial pressure (MAP) only modestly increased (by ~13%). In contrast, the modified hemoglobin solution caused less increase in SVR (~14%) and did not alter renal hemodynamics or blood pressure. We also compared the ability of the unmodified and crosslinked hemoglobins to inactivate nitric oxide (NO) *in vitro* using three separate assays; platelet NO release, NO-stimulated platelet cGMP production and EDRF-mediated inhibition of platelet aggregation. The unmodified hemoglobin inactivated NO to a markedly greater extent than the o-raffinose crosslinked hemoglobin in all three assays. Thus, chemical crosslinking with o-raffinose reduces the systemic and renal vasoconstriction as well as the NO-inactivating effects of hemoglobin. We suggest that the change in the vasoactive effects of unmodified hemoglobin induced by o-raffinose crosslinking is related to the associated reduction in NO-inactivation.

KEY WORDS: Vascular resistance, blood pressure, renal blood flow, glomerular filtration rate, cardiac output

INTRODUCTION

Hemoglobin-based oxygen carrying solutions (HBOC's) have a number of potential advantages as blood substitutes. They can be produced in large volumes, stored for prolonged periods and administered rapidly, without need for typing and crossmatching. These solutions can also be sterilized by pasteurization, reducing the risk of contamination by viruses and other infectious agents (1-4). A major challenge to the development of a safe red cell-free hemoglobin-based oxygen carrier has been the well recognized nephrotoxicity of crude hemolysate (5-8). However, three fundamental observations, made during the past few decades, have markedly reduced the toxicity and increased the efficacy of hemoglobin solutions (8).

First, in 1967, Rabiner et al (9) demonstrated that the presence of red blood cell membrane fragments (red cell "stroma") contribute substantially to the toxicity and procoagulant activity of crude red cell hemolysates. This observation spurred the development of effective techniques to purify hemoglobin, a process that reduced, but did not eliminate, the toxicity of hemoglobin solutions (10). Highly purified solutions of hemoglobin (which have become known as "stroma free" hemoglobin (SFH) solutions) can now be prepared in large volumes (2).

A second important innovation was that hemoglobin molecules can be chemically modified to prevent the excretion of hemoglobin in the urine. Native hemoglobin (~64,500kD), when released from red cells, dissociates into dimers (~32,250kD) that are readily filtered at the glomerulus (11). The presence of hemoglobin within the tubular lumen contributes substantially to hemoglobin-associated nephrotoxicity by causing intratubular obstruction and oxidant injury to renal epithelial cells. (5-7, 12-14). Chemical crosslinking of dimers into tetramers and/or oligomers is one effective form of modification that reduces the ability of hemoglobin to cross the glomerular basement membrane and gain access to the renal tubules and substantially reduces nephrotoxicity

(1-3, 8). An added advantage of chemical modification is that it prolongs the half-life of the hemoglobin in the circulation by preventing elimination in the urine and by slowing the rate of diffusion of hemoglobin from the circulation into the interstitial space (1-3, 8).

A third discovery, essential for the efficacy of SFH solutions as effective oxygen carriers, was the use of chemical modification to optimize the oxygen affinity of SFH. Within the human red cell, the presence of 2,3,diphosphoglycerate (2,3-DPG) normally maintains the normal affinity of human hemoglobin for oxygen. Red cell-free hemoglobin loses this interaction with 2,3 DPG so that unmodified human SFH solutions have a very high oxygen affinity. Benesch and Benesch (15) were the first to demonstrate that chemical modification methods can also be used to reduce the oxygen affinity of the SFH for oxygen, resulting in an oxygen carrier that will effectively release oxygen at the physiologic pO_2 present in tissues.

However, despite the development of increasingly efficient and effective ways of removing red cell stroma and of cross-linking hemoglobin, many SFH solutions have continued to exhibit vasoconstrictor activity with resultant hypertension and reduced blood flow to various organs, including the heart, lung and kidney (8, 10, 16-18). There is substantial evidence that these vasoactive properties of purified and modified SFH solutions are due, at least in part, to the well established ability of hemoglobin to bind and inactivate nitric oxide (NO) (19-23).

This study was designed to evaluate systemic and renal hemodynamic effects of a highly purified hemoglobin solution that has been chemically crosslinked with o-raffinose to form oligomers of hemoglobin with a weight-average molecular weight of 230,000 (24). We have directly compared the hemodynamic effects of modified o-raffinose crosslinked hemoglobin with the highly purifed unmodified human hemoglobin Ao from which the crosslinked hemoglobin was made. We demonstrated that the unmodified SFH causes a

severe increase in systemic vascular resistance and intrarenal vasoconstriction, and markedly reduces both cardiac output as well as renal function. In striking contrast, the o-raffinose crosslinked hemoglobin has no deleterious effect on cardiac function, glomerular filtration rate (GFR), or renal vascular resistance, and only modestly increases systemic vascular resistance. We also provide *in vitro* evidence that the effects of o-raffinose crosslinking on vasoactivity is associated with differences in the degree to which the modified and unmodified forms of the SFH solutions inactivate nitric oxide (NO).

METHODS

A. Reagents

Human serum albumin, sodium nitrite, trichloroacetic acid, trypan blue, Sepharose 2B, adenosine 5'-diphosphate (ADP), glutathione (GSH) and Nω-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Co. (St. Louis, MO). Microcarrier beads (Cytodex 3) were obtained from Pharmacia, Uppsala, Sweden. Cyclic GMP enzyme immunoassay kits were purchased from Cayman Chemical Co. (Ann Arbor, MI). Inactin (thiobarbital sodium) was purchased from Research Biochemicals International (RBI) (Natick, MA).

B. Resuscitation solutions used for exchange transfusion

In animals exchanged with whole blood, the blood removed during the exchange was anticoagulated with heparin and administered back to the same animal. Human serum albumin (6g/dL) in Ringer's lactate was used as a non-hemoglobin-containing colloid solution. Human purified stroma-free hemoglobin (SFH) solutions were provided by Hemosol, Inc. (Toronto, Canada). All hemoglobin solutions were prepared in Ringer's lactate solution.

1) Methods used to produce the SFH solutions

a) Unmodified human hemoglobin Ao (HbAo): Outdated human red cells are pooled, washed to remove plasma proteins, and lysed by gentle osmotic shock to minimize release of stromal components. The crude hemoglobin is separated from red cell ghosts by membrane microfiltration. The hemoglobin is converted to carbonmonoxy-hemoglobin, and pasteurized for 10 hours at 62°C. Further purification is achieved using both anion- and cation-exchange chromatography to remove basic and acidic protein contaminants and to further reduce lipid and encapsulated virus contamination. A proprietary self displacement chromatography process, developed by Hemosol Inc., is used to prepare large volumes of purified HbAo. (25). The purity of the HbAo, confirmed by several methods, is greater than 99%. No red cell membrane proteins are detectable. The

combined phosphatidylethanolamine and phosphatidylinositol content of purified HbAo is <2μg/g hemoglobin (25, 26)

b) SFH solutions crosslinked with o-raffinose

i) o-raffinose crosslinked SFH solution #1 (Hemolink™): The deoxygenated HbAo is crosslinked through oxidation of the trisaccharide raffinose (25, 27). The aldehydes initially form imine, or Schiff base linkages with amino groups within and between individual hemoglobin molecules. These reversible bonds are then stabilized by chemical reduction using dimethylamino borane (28). This reductive alkylation is irreversible and covalently crosslinks the two αβ dimers between amino groups within the 2,3-DPG binding pocket to form stable tetramers. In addition o-raffinose reacts with surface amino groups of these tetramers to create intermolecular linkages producing a stabilized oligomeric hemoglobin with a weight-average molecular weight of 230,000 (ranging from 128,000 to 500,000). Methemoglobin is also reduced (from Fe^{3+} to Fe^{2+}) during this treatment. Unreacted aldehydes are simultaneously reduced to alcohols to prevent further crosslinking. Excess reagents and byproducts are removed by diafiltration and the final solution formulated in the oxygenated state, in Ringer's lactate (USP) for injection (24).

The o-raffinose solution #1 consists predominantly of a polymeric form of hemoglobin with a weight-averaged molecular weight of approximately 230,000 (Table I). However, a substantial proportion of hemoglobin is in the tetrameric, 64Kd form (31.8%) and a small proportion of unmodified dimers of hemoglobin (32Kd) remains following diaflitration (Table I).

ii) o-Raffinose crosslinked SFH solution #2: In order to determine the extent to which the residual dimeric (32kD) hemoglobin affected the hemodynamic effects of the modified SFH, o-raffinose crosslinked hemoglobin was processed to remove most of the remaining dimeric hemoglobin, leaving only 0.6% of

hemoglobin in the dimerized form. This solution, called o-raffinose SFH solution #2 still contained substantial amounts (18.1%) of tetrameric hemoglobin (Table I).

iii) o-Raffinose crosslinked SFH solution #3: In order to determine the extent to which the residual tetrameric (64kD) hemoglobin moiety affected the hemodynamic effects of the modified SFH, o-raffinose crosslinked hemoglobin was processed to remove most of the tetrameric hemoglobin as well as most of the dimeric hemoglobin. In this solution, called o-raffinose crosslinked SFH solution #3, very little of the total hemoglobin was present in either the dimeric (0.3%) or tetrameric (1.8%) forms (Table 1).

2. The characteristics of the resuscitation fluids.

The proportion of hemoglobin in the oligomeric, tetrameric and dimeric forms in the different o-raffinose SFH solutions is shown in Table I. The methods used to determine the proportion of different the molecular weight species of hemoglobin in the o-raffinose SFH solutions are described elsewhere (25).

The human serum albumin (HSA), unmodified (Ao) SFH, and the three o-raffinose crosslinked solutions of SFH all had a comparable oncotic pressure of 20-24mmHg (Table II). Oncotic pressures were determined using a Wescor oncometer (Model 4400).

The proportion of hemoglobin in the form of methemoglobin was comparable in all SFH solutions (4-5%) (Table II). Oxy- and methemoglobin were measured with a CO-oximeter (Model 282, Instrumentation laboratory, Lexington, MA.). The p50 (measured with a Hemoxanalyser (29)) of the unmodifed hemoglobin was very low (14.6mmHg) (Table 1I). The crosslinking procedure substantially reduced the oxygen affinity of all o-raffinose solutions to a comparable extent (p50 values ~44mmHg) (Table II).

C. *In vivo* rat protocols:

1) Surgical procedure

Male Sprague-Dawley rats, weighing between 275 and 350g, were used for all experiments. Rats were fed regular Purina rat chow (Purina Mills, Chicago, IL) and allowed free access to water. Anesthesia was induced with an intraperitoneal injection of Inactin (thiobarbital sodium) (110mg/kg). Rats were placed on a thermostatically controlled heated table. Body temperature was monitored via a temperature probe in the carotid artery and maintained between 36°C and 38°C. A tracheotomy was performed with the use of polyethylene (PE-240) tubing, and both femoral arteries were cannulated with PE-50 tubing, one for blood pressure monitoring and the other for blood sampling. A bladder catheter (PE-90) was placed via a suprapubic incision for urine sampling. The right internal jugular vein was cannulated with two PE-50 catheters. Inulin and PAH were infused via one catheter. The second catheter was advanced into the superior vena cava. This catheter was used for infusion of the resuscitation fluids (blood, SFH and albumin), and was also used for cardiac output measurements as described below.

2) Hemodynamic measurements and assays.

a) Cardiac output was measured with a Cardiomax II-R instrument (Columbus Instruments Corp., Columbus, OH.) using the thermodilution technique as previously described (30). The right carotid artery was cannulated with a thermistor for measurement of thermodilution cardiac output curves. A #1.5 French thermosensitive microprobe (Columbus Instruments Inc., Columbus, OH) was placed into the right carotid artery. The microprobe was advanced along the right carotid artery until resistance was provided by the wall of the aorta at which point the probe was withdrawn by 1/16 of an inch. Using this technique, the tip of the probe was positioned in the aortic arch just distal to the aortic valve (30).

Cardiac output was measured by injecting 200µl of 5% dextrose in water at 20±2°C into the right superior vena cava catheter using a Hamilton syringe. Within 10-15 seconds, a

readout of cardiac output (CO), stroke volume, and heart rate was obtained. The determination of CO by this method is dependent on an integration of the area under the curve of the temperature change. Stroke volume is calculated by the Cardiomax II-R from the measured cardiac output and heart rate. The Cardiomax II-R also provided a constant read out of heart rate and mean arterial pressure via the femoral artery catheter and of body temperature via the temperature sensitive probe placed in the aortic arch (30).

b) Measurement of glomerular filtration rate (GFR) and renal plasma flow (RPF). Glomerular filtration rate (GFR) and effective renal plasma flow were determined by the renal clearance of inulin- [carboxyl-^{14}C] and aminohippuric acid P-[glycyl-2-^{3}H] ,respectively (New England Nuclear, Boston, MA). The [^{14}C]-inulin was infused at a rate of 0.06 μCi/min and the [^{3}H]-PAH at a rate of 0.23 μCi/min. Blood samples, obtained at the midpoint of each clearance period, were centrifuged and 5μl samples of plasma and urine containing both [^{14}C] and [^{3}H] were added separately to 3 ml liquid scintillation fluid and counted in a Packard Tri-Carb (1600TR) liquid scintillation analyzer for 10 minutes (30). In order to separate the spectra of the two radionuclides, the technique of full-spectrum dual-disintegrations-per-minute (DPM) counting was used as previously described (30). Urinary and plasma electrolyte concentrations were measured using ion specific electrodes.

c) Calculations of renal blood flow (RBF), filtration fraction (FF), systemic vascular resistance (SVR), renal vascular resistance (RVR), and fractional excretion of sodium (FeNa) and potassium (FeK).

GFR and PAH clearances were calculated using standard formulae. Renal plasma flow (RPF) was calculated from the clearance of PAH assuming a PAH extraction of 80% (31). RBF was calculated as RPF/(1 - hematocrit). The FF is the fraction of plasma flow filtered at the glomerulus ((GFR/RPF)X100)). SVR was calculated by dividing MAP by CO. RVR was calculated by dividing MAP by RBF. FeNa and FeK were calculated by determining the

concentration ratios of urine:plasma for Na and K and then by dividing these ratios by the concentration ratio of urine:plasma for inulin.

2. Exchange transfusion protocol.

The rats were subjected to a 20% exchange transfusion by removing 4 ml whole blood/300 g body weight from a femoral artery over 15 minutes. Then animals were divided into separate groups to receive 4 ml/300 g body weight of six different resuscitation fluids via a jugular venous line over a 10 minute period. The six solutions administered were: 1) whole blood (n= 16); 2) Human serum albumin (HSA) 6g/dL in Ringer's lactate; 3) unmodified (Ao) SFH (n=15); 4) o-Raffinose SFH #1(n=24); 5) o-Raffinose SFH #2 (n=10); and 6) o-Raffinose SFH #3 (n=10)(Table I)

A separate group of rats (n=5) were subjected to sham surgery. These rats were anesthetized and exposed to the same operative maneuvers as the other rats except that no blood was removed and no resuscitative fluid was administered. After an equilibration period of 30 minutes, four 20-minute clearance periods were obtained in all groups for measurement of MAP, CO, heart rate (HR), GFR, and renal plasma flow (RPF). Renal blood flow (RBF), renal vascular resistance (RVR), systemic vascular resistance (SVR), fractional sodium excretion (FeNa) and fractional potassium excretion (FeK) were calculated as described above.

3. Experimental protocols to compare plasma levels and urine excretion of unmodified and modified SFH solutions.

In separate groups of animals, we determined the plasma levels and urine excretion rates of Ao SFH and o-raffinose SFH #1 . Two groups of rats were exchange transfused with either Ao SFH (n=4) or o-raffinose SFH solution #1 (n=5) using the same methods described above. Heparanized blood was obtained by heart puncture 10 minutes after infusion. The blood was centrifuged and the plasma frozen at -70°C for measurement of

hemoglobin levels. Two separate groups of rats were also exchange-transfused with either Ao SFH (n=8) or o-raffinose SFH #1 (n=5), and urine was collected for 110 minutes starting immediately after completion of the infusion of SFH. At the end of the urine collection, heparinized blood was obtained by heart puncture for hemoglobin measurement. The volume of urine excreted over the 110 minutes was measured and the urine frozen for measurement of hemoglobin levels. Total free hemoglobin in plasma and urine was measured using a dual beam spectrophotometric assay as previously described (32). Using this assay the lowest level of detection of hemoglobin is 1 mg/dl.

D. *In vitro* assays of hemoglobin-nitric oxide (NO) interactions

1) Preparation of the EDRF congener S-nitrosoglutathione (SNO-GSH)

SNO-GSH was prepared by reacting freshly prepared solutions of GSH with sodium nitrite at acidic pH, as previously described (33, 34). SNO-GSH was prepared within 10 minutes of use, kept at 4°C, and diluted as necessary into aqueous buffer immediately before addition to assay systems.

2) Preparation of platelet-rich plasma (PRP)

Peripheral blood was drawn from healthy adult human volunteers who had not consumed acetylsalicylic acid or any other platelet inhibitor for at least seven days. The first 2 mL of blood drawn were discarded. Blood was then drawn into a sodium citrate anticoagulant. The citrated blood was centrifuged (150 x g, 15 minutes, 22°C) and the supernatant (PRP) was removed for further processing.

3) Preparation of Gel-Filtered Platelets

Gel-filtered platelets (GFP) were obtained by passing PRP over a Sepharose-2B column in Tyrode's-Hepes buffered saline. Tyrode's-Hepes-buffered saline (HBS) consisted of 140 mM NaCl, 6 mM *N*-[2-hydroxyethyl]-piperazine-*N*-[2-ethane-sulfonic acid], 2 mM Na_2HPO_4, 2 mM $MgSO_4$, 0.1% dextrose, and 0.4% BSA (pH 7.4,). Platelet counts were determined using a Coulter Counter, model ZM (Coulter Electronics, Hialeah, FL). Platelets were adjusted to $1.5x10^8$ platelets/ml by the addition of Tyrode's-Hepes-buffered saline.

4) Measurement of platelet aggregation

Aggregation of GFP (0.2 ml) was monitored using a standard nephelometric technique in which changes in light transmittance are recorded as a function of time (35). Aggregations were induced by adding 5 μM ADP, and the absolute change of light transmittance was recorded in a four-chamber aggregometer (BioData, Hatboro, Pennsylvania). GFP were incubated for one minute at 37°C with either i) S-nitroso-glutathione (SNO-GSH), ii) bovine endothelial cells on beads (ECBs), iii) SNO-GSH + SFH or iv) ECBs + SFH prior to the addition of ADP.

5) Measurement of platelet cyclic guanosine monophosphate (cGMP) production. Trichloroacetic acid (final concentration, 5% vol/vol) was added to GFP. Samples were vortexed, placed on ice, and centrifuged at 1500xg for 10 minutes at 4°C. The supernatant was extracted with diethyl ether five times and assayed for cGMP by an enzyme-linked immunoadsorbent assay (ELISA) methodology using cGMP antiserum (Cayman Chemical Co., Ann Arbor, MI).

6) Measurement of platelet NO production

We adapted a NO-selective micro-electrode (Inter Medical Co., Ltd. Nagoya, Japan) (36) for use in a standard platelet aggregometer (Payton Associates, Buffalo, NY) in order to monitor platelet NO production and aggregation simultaneously. The NO monitoring device consists of a headstage amplifier with a built-in power supply and two electrodes. The working electrode (0.2 mm diameter, made from Pt/Ir alloy) and counterelectrode were inserted using a micro-manipulator into an aggregometer microcuvette containing a stir bar. The aggregometer, electrode, and headstage amplifier were housed in a Faraday cage to reduce electrical interference. The electrode current increased linearly from 10 to 350 pA over a range of NO concentrations. All experiments were conducted at constant temperature (37°C). Production of NO was determined by integrating the area under the response curve. Diethylamine NONOate was used to derive a standard curve which was linear over the range of 10-500 nM with a typical correlation coefficient of 0.95.

7) Determination of EDRF-induced inhibition of platelet aggregation

a) Preparation of endothelial cell cultures on microcarrier beads (ECBs)

Bovine aortic endothelial cells were isolated and then grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum by established techniques (37). Cells were grown to confluence on a micocarrier system of collagen-coated spherical beads (Cytodex) as previously described (37). The bovine endothelial cell-coated beads (ECBs) were exposed to 30 μM acetylsalicylic acid for 1 h at 37°C to inhibit cyclooxygenase activity and prostanoid production, and then washed three times in HBS immediately before use. Endothelial cells were checked for viability by trypan blue exclusion. ECBs were drawn up and permitted to settle in a packed volume of approximately 500 μl in a 1-ml Eppendorf pipette tip as previously described (37).

b) Measurement of the effect of unmodified and modified SFH on ECB-induced inhibition of platelet aggregation: Bovine aortic endothelial cells (BAECs) grown on Cytodex beads (ECBs) were incubated with or without 1μM unmodified or*O*-Raffinose cross-linked SFH solutions for 5 minutes. Then, GFP was added and the suspension stirred in an aggregometer for 5 minutes. The ECBs were allowed to settle, the GFP removed, and aggregation induced with 5μM ADP and the extent of platelet aggregation determined as descibed above. Cytodex beads without endothelial cells served as control and had no effect on platelet aggregation. Also, pre-incubation of ECBs with the NO synthase inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME), before addition of GFPs abrogated inhibition of platelet aggregation, clearly demonstrating that EDRF is the platelet inhibitory factor in this asay.

E. Statistics

All data are expressed as mean ± SE. Measurements obtained during all four observational periods were averaged and represent systemic and renal hemodynamic data for the 80 minutes following exchange transfusion. All variables of the seven groups of rats were compared by ANOVA. In those variables in which ANOVA demonstrated a difference

amongst the groups, the Duncan's or the Newman-Keuls test was performed. Statistics were done using SAS 6.12 statistical software. Values for individual clearance periods for systemic vascular resistance were compared using the student t-test with the Bonferroni correction. A p value of <0.05 was considered significant.

RESULTS

A. Exchange-transfusion experiments

1) Effects of exchange-transfusion or sham surgery on hematocrit.

The hematocrits were measured at the midoint of each of the four observational periods and meaned. There was no difference between the hematocrit of sham operated rats (46.7±0.2%) and rats exchange transfused with whole blood (47.0±0.5). The hematocrits of animals exchange transfused with albumin (34.0±0.5), SFH Ao (38.2±0.6%), and o-raffinose cross-linked SFH solutions #1 (35.5±0.5), #2 (34±1.0%) and #3 (33.2±0.6) were lower than those of sham operated rats and rats exchange-transfused with whole blood ($p<0.01$)(Figure 1). The hematocrit of the unmodified (Ao) SFH exchanged rats was higher than that of rats exchanged with albumin or the o-raffinose cross-linked SFH solutions ($p<0.01$).

2. Effects of exchange transfusion on systemic hemodynamics.

MAP in rats exchange transfused with unmodified hemoglobin (Ao) was increased by ~15% compared to blood exchanged animals ($P<0.01$ vs blood group)(Table III). MAP in rats exchanged transfused with o-raffinose crosslinked SFH solution #1 was increased by ~10%($p<0.01$ vs blood group) and comparable to the change in MAP induced by hemoglobin Ao (Table III). The MAP in rats exchanged-ransfused with o-raffinose crosslinked SFH solution #2 and o-raffinose crosslinked SFH solution #3 were not significantly different from MAP in whole blood exchanged animals (Table III).

The unmodified SFH markedly reduced cardiac output by ~21% below that of rats exchanged with whole blood. In contrast, the cardiac outputs of rats exchanged with all the ox-raffinose solutions were comparable to the cardiac output meausred in blood-exchanged rats. The albumin solution increased cardiac output compared to blood exchanged animals by ~30% ($P<0.01$)(Table III).

The albumin solution caused a fall in SVR below blood exchanged rats (Table III). The unmodified (Ao) SFH solution resulted in a marked (~46%) increase in SVR above that inanimals exchanged with whole blood ($P<0.01$)(Figure 2, Table III). The o-raffinose crosslinked SFH solution #1 increased SVR to a substantially lesser extent than Ao SFH (by ~15%) ($P<0.01$) (Figure 2, Table III). The SVR in animals exchanged with o-raffinose crosslinked SFH solution #2 and o-raffinose crosslinked SFH solution #3 was ~12% higher than SVR in blood exchanged rats, although this difference was not statistically significant (Table III). SVR was sustained during each of the four observational periods in rats exchanged with whole blood, Ao SFH or o-raffinose crosslinked SFH solution #2 (Figure 2).

3. Effects of exchange transfusion or sham surgery on renal function (GFR), intrarenal hemodynamics and electrolyte excretion.

The unmodified hemoglobin Ao markedly reduced GFR and renal blood flow below that of animals exhanged with whole blood, albumin, and o-raffinose crosslinked SFH solutions ($p<0.01$)(Table III). Calculated RVR was reduced in albumin exchanged rats below blood exchanged rats. RVR was markedly increased (almost two fold) in Ao hemoglobin exchanged rats compared to RVR in whole blood exchanged animals. The RVR in all three *O*-raffinose exchanged rats was not different from blood exchanged animals.

Fractional excretion of sodium (FeNa) and potassium (FeK) was higher in animals exchanged with albumin, unmodifed SFH and the o-raffinose crosslinked SFH solutions compared to blood exchanged animals ($p<0.01$)(Table V).

4. Plasma and urine hemoglobin levels.

Both the Ao SFH and the o-raffinose crosslinked SFH solution #1 reached comparable concentrations in plasma when measured 10 minutes after exchange transfusion (Table VI). The concentration of both SFH solutions fell over the the 110 minute period of observation but the plasma level of the unmodified SFH fell to a far greater extent than that of the o-raffinose crosslinked SFH (Table VI). A substantial amount of the unmodified hemoglobin Ao was excreted in the urine (282±0.1ug/min). The total amount of unmodofied SFH eliminated by the kidney represents at least 35% of the administered load. In contrast, in rats given o-raffinose crosslinked SFH, no hemoglobin was detectable in the urine.

B. Interactions between the hemoglobin solutions and nitric oxide *in vitro*.

1. Effect of hemoglobins on platelet release of NO.

Platelets activated with 5μM ADP produce NO, which diffuses from the platelets and inhibits the recruitment of additional platelets to the growing platelet thrombus (38). We have adapted a microelectrode methodology to measure platelet NO production in real time during the platelet aggregation response. NO bound by hemoglobin is not detected by this method.

Gel-filtered platelet aggregation was induced with 5μM ADP in the absence or presence of 1μM heme in the form of unmodified SFH or o-raffinose crosslinked SFH solutions #1, #2 or #3. As shown in Table VII, the unmodified hemoglobin inhibited NO released by aggregating platelets almost completely (by ~99%). The o-raffinose crosslinked SFH solutions also reduced NO release, but did so to a far lesser extent than the unmodified

SFH (by ~80%). NO release in the presence of the three o-raffinose crosslinked SFH solutions was comparable (Table VII).

2. Effect of SFH solutions on platelet cGMP production induced by SNO-GSH.

Gel-filtered platelets were incubated with 10 μM SNO-Glu for 5 minutes. Platelet proteins were then precipitated with trichloroacetic acid and cGMP content determined in the supernatant. Unmodified SFH markedly suppressed platelet cGMP below control values (Table VIII). In contrast, all O-raffinose cross-linked SFH solutions had only a modest effect on cGMP production which was not statistically different from control values (Table VIII).

3. Effect of hemoglobin solutions on EDRF inhibition of platelet aggregation.

Bovine aortic endothelial cells (BAECs) grown on Cytodex beads (ECBs) were incubated with or without 1μM unmodified or o-raffinose crosslinked SFH solutions for 5 minutes. Then, GFP was added and the suspension stirred in an aggregometer for 5 minutes. The ECBs were allowed to settle, the GFP removed, and aggregation induced with 5μM ADP. As shown in Figure 3, unmodified SFH reversed the inhibition of platelet aggregation by EDRF released from ECBs with the extent of aggregation increasing 223±43% above control (Figure 3). In contrast, the o-raffinose crosslinked SFH solutions had a far more modest effect on EDRF inhibition of platelet aggregation, increasing the extent of aggregation by only 30-60%. There was no significant difference among the three o-raffinose crosslinked SFH solutions (Figure 3).

DISCUSSION

Many different preparations of SFH have been developed and evaluated by a diverse array of research groups in academic centers, private companies as well as the military (1, 3, 8). While all of these SFH solutions are subjected to purification techniques with removal of most of the non-hemoglobin contaminants, the method and efficacy of purification differs substantially amongst SFH solutions. The presence of some non-hemoglobin contaminants are thought to contribute to the vasoactive effects of some of these products

(16). At present there is still some uncertainty regarding the extent to which the hemoglobin molecule itself contributes to the toxicity of SFH solutions (2, 8, 39).

The major purpose of this study was to examine the influence of one available method of chemical crosslinking on the vasoactivity of the hemoglobin molecule itself. This represents the first study in which the hemodynamic and renal effects of a modified SFH solution and its unmodified precursor are directly compared. We compared the effects of o-raffinose crosslinked hemoglobin and unmodified, highly pure SFH Ao which was used to make the crosslinked SFH. Since both unmodified and modified solutions are of comparable purity (25), any differences in cardiac, renal, or vascular effects cannot be ascribed to differences in the concentration of non-hemoglobin contaminants.

The hematocrit of rats exchange transfused with whole blood was the same as sham operated rats (Figure 1). The hematocrit was reduced to the same degree in rats exchanged with HSA and each of the three o-raffinose crosslinked solutions (Figure 1). However, while the hematocrit was markedly reduced in rats exchanged with the unmodified SFH, it was significantly higher than in the groups exchanged with HSA and all the o-raffinose crosslinked SFH solutions (Figure 1). Since the unmodified SFH is rapidly cleared from the plasma (Table VI), the loss of the colloid from the circulation likely leads to diffusion of plasma water from the vascular bed with resultant increase in the hematocrit. It is also possible that the intense vasoconstriction induced by the unmodified SFH increases the filtration of plasma water into the interstitial space as a result of altered Starling's forces.

We demonstrated marked differences in the systemic and intrarenal effects betrween modified and unmodified hemoglobin solutions. The unmodified hemoglobin (SFH Ao) results in a marked elevation in systemic vascular resistance (SVR) of ~45% when compared to the effects of exchange with whole blood (Table III). MAP is also increased by Ao SFH but, by comparison with the change in SVR, this increase is relatively modest (by

~14%)(Table III). The marked proportional difference in the severity of the increase in MAP and SVR is accounted for by the profound depressant effect of Ao SFH on cardic output (Table III). We have not examined the extent to which the reduction in cardiac output induced by Ao SFH is the possible result of myocardial ischemia (due to vasoconstriction of the coronary vasculature) and/or direct suppression of myocardial contractility. Ao SFH also induced marked constriction of the renal vasulature with resultant reduction in both GFR and RBF (Table IV).

While the o-raffinose crosslinked SFH solution #1 also increases SVR, this effect is relatively modest and substantially less than the increase in SVR induced by the Ao SFH (Table III). In addition, o-raffinose crosslinked SFH solution #1 has no deleterious effect on cardiac output so that the increase in MAP induced by o-raffinose crosslinked SFH solution #1 is proportionately comparable to the modest increase in SVR induced by this modified SFH solution. Also, unlike the unmodified SFH Ao, o-raffinose crosslinke SFH solution #1 has no effect on the GFR, renal blood flow, or RVR when compared to rats exchanged with whole blood (Table III and IV). Thus, our data demonstrate that the process of o-raffinose crosslinking markedly alters the vasoactive properties of the hemoglobin molecule itself. Adverse effects on MAP and SVR are markedly attenuated and reductions in cardiac and renal function completely eliminated.

All SFH solutions as well as the HSA (unmodified as well as modified) increased the fractional excretion of sodium (FeNa) and potassium (FeK) when compared to whole blood-exchanged rats and to a comparable extent (Table V). The cause of this increase in fractional electrolyte excretion is probably multifactorial, but compared to rats receiving whole blood, those given either SFH solutions or HSA received about twice the volume of red-cell free electrolyte solution. This augmented water and electrolyte load likely contributed to the diuresis, natriuresis and kaliuresis associated with all these solutions.

We also examined the extent to which the modest effects of the o-raffinose cross-linked SFH solution #1 on SVR and MAP are due to the presence of some smaller molecular weight moieties (dimers and tetramers) of hemoglobin in this solution. We compared the effects of o-raffinose SFH in which the concentration of the dimeric hemoglobin (o-raffinose crosslinked SFH solution #2) or dimeric as well as tetrameric hemoglobin (o-raffinose crosslinked SFH solution #3) was substantially reduced (Table I). The effects of these solutions on MAP, cardiac output, SVR, RVR and GFR are not different from those of the o-raffinose crosslinked SFH solution #1. Thus, efforts to remove the smaller molecular weight hemoglobin molecules present in the o-raffinose crosslinked SFH solution #1 provided no substantive benefit in systemic or renal hemodynamics.

We have also found that the retention time of the unmodified SFH Ao in the circulation is extremely short. The rapid fall in plasma levels after infusion of Ao SFH (Table VI) is likely due to rapid leakage out of the vascular space into interstitial fluid as well as to excretion in the urine. The increased plasma retention time of the polymerized solution and the absence of appearance of the modified hemoglobin in the urine is likely due to the increased size of oligomerized hemoglobin, which prevents loss of hemoglobin in the urine and slows passage of the hemoglobin across capillary walls.

We did additional studies to investigate the mechanism/s responsible for the striking reduction in vasoactivity associated with o-raffinose crosslinked SFH. One mechanism by which the hemoglobin molecule may potentially induce systemic and intrarenal vasoconstriction is by inactivating or binding nitric oxide (NO)(8). The ability of hemoglobin to inhibit endothelium-dependent vasodilation (EDRF) was demonstrated (19) long before endothelial release of NO was shown to account for this activity (40). Subsequently, the interactions between hemoglobin and NO have been well characterised (20-22, 41). The binding affinity of hemoglobin for NO is much higher than for either O_2 or CO (20). Hemoglobin can interact with NO in a number of different ways. Firstly, NO can

convert oxyhemoglobin (Hb[FeII]O_2) to methemoglobin (Hb[FeIII]); as a result of this reaction NO is inactivated and converted to nitrite and nitrate. Secondly, NO can bind to the heme group of deoxyhemoglobin (Hb[FeII]) forming a highly stable complex(20). Finally, some evidence suggests that NO can bind to sulfhydryl groups on the globin chain of hemoglobin (42).

The constitutive production of endothelial-derived NO plays an important role in regulating normal vascular tone and blood pressure (43). Thus, inhibition of NO activity by hemoglobin would be expected to cause systemic vasoconstriction and hypertension as well as intrarenal vasoconstriction and a reduction in GFR (17, 21-23, 43).

In order to determine if the reduced vasoactivity of the o-raffinose crosslinked SFH solutions is related due to an alteration in the binding of NO, we compared the effects of the unmodified and o-raffinose crosslinked SFH solutions on NO activity *in vitro* . We used a number of different assay systems, including platelet NO production (Table VII), platelet cGMP production (Table VIII) and EDRF-induced inhibition of platelet aggregation (Figure 3). All these assays indicate that all three o-raffinose crosslinked SFH solutions have substantially less NO binding and/or inactivating properties than the unmodified precursor SFH solution. It is likely that the more modest effects of modified SFH compared to unmodified SFH on systemic vasocontriction, cardiac output and renal function are due, at least in part, to the greater bioavailablity of NO.

The mechanisms whereby o-raffinose cross-linking of hemoglobin appears to modulate the interaction of the hemoglobin with NO remains to be determined. However, data from Rogers and colleagues (44) demonstrate that cross-linking of hemoglobin tends to selectively modify ligand interactions and redox kinetics in a manner consistent with a more open heme pocket in the globin tertiary structure. In addition, Sharma et al (45) have shown that the presence of an axial water molecule in the ligand binding site of ferric

hemoglobin A substantially attenuates the cooperativity in its reactions with NO. Taken together, these observations suggest the possibility that cross-linking hemoglobin changes the conformation of the heme binding site, and enhances hydration rates, thereby decreasing reactivity toward NO.

In summary,unmodified SFH causes marked systemic and intrarenal vasoconstriction, and a profound reduction in both cardiac output and GFR. In marked contrast, the o-raffinose crosslinked SFH solutions increase SVR only modestly and have no effect on cardiac output, GFR or renal hemodynamics. Since this is the first *in vivo* study that has compared unmodified and modified hemoglobins of identical purity, we are able to clearly establish that the hemoglobin molecule itself has vasoconstrictor properties that are independent of and likely additive to the effects of contamination by red cell stroma. We have also shown that one form of hemoglobin modification, (using o-raffinose to cross-link hemoglobin into oligomers), substantially ameliorates the vasoconstrictor effects and eliminates the adverse effects of unmodified hemoglobin of cardiac and renal function. The mechanism responsible for these beneficial effects of cross-linking have not been directly examined *in vivo*. However, *in vitro* studies demonstrate that the cross-linked hemoglobin inactivates NO to a substantially lesser extent than unmodified hemoglobin. On the basis of these findings we suggest that the differences in the hemodynamic effects of the unmodified and o-raffinose crosslinked SFH solutions is related, at least in part, to differences in the extent to which NO is inactivated by these two SFH solutions *in vivo*. The development of an SFH solution with no acute nephrotoxic or cardiotoxic effects is an encouraging advance in the development of a therapeutically useful hemoglobin- based oxygen carrying solution.

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TABLE I

Proportion of different molecular weight moieties of hemoglobin in the three o-raffinose solutions of SFH

	Hemoblobin oligomers (m.w.= ~230Kd)	Hemoglobin tetramers (m.w.=64Kd)	Hemoglobin dimers (m.w.=32Kd)
o-raffinose crosslinked SFH #1	63.8%	31.8%	2.6%
o-raffinose crosslinked SFH #2	81.1%	18.1%	0.6%
o-raffinose crosslinked SFH #3	96.9%	1.8%	0.3%

TABLE II

Characteristics of the human serum albumin (HSA) and SFH preparations

	HSA	Ao SFH	o-raffinose SFH (Solution #1)*	o-raffinose SFH (Solution #2)	o-raffinose SFH (Solution #3)
Concentration (g/dL)	6.0	6.9	9.8	10.0	9.9
Colloid oncotic pressure (mmHg) (mmHg)	20	24	22	23	22
Methemoglobin (%)	n/m*	4.7	4.1	5.0	4.9
p50 (mmHg)	n/m*	14.6	43	44.9	44.1

*O-raffinose cross-linked SFH #1 = Hemolink™; † n/m=not measured

TABLE III

Comparison of the effects of sham surgery and exchange transfusion with blood, 6% albumin, or SFH solutions on mean arterial pressure (MAP), cardiac output (CO) and systemic vascular resistance (SVR)

	MAP (mmHg)	CO (ml/min/300g)	SVR (mmHg/ml/min/300g)
Blood group (n=16)	109±2	111±3	0.99±0.04
Sham group (n= 5)	111±2	108±3	1.03±0.02
Cross-linked SFH groups:			
o-raffinose crosslinked SFH #1 (n=24)	119±5*	108±2	1.13±0.03*
o-raffinose crosslinked SFH #2 (n=10)	113±5	102±3	1.10±0.04
o-raffinose crosslinked SFH #3 (n=10)	114±4	106±4	1.09±0.05
Unmodified SFH (n=15)	124±2*	89±4*†	1.43±0.06*†
Albumin group (n=10)	104±2*†	144±5*†	0.73±0.03*†

*p<0.01 compared to sham operated group or rats exchange-tranfused with whole blood
†=p<0.01 compared to groups exchanged with *O*-raffinose crosslinked solutions #1 (Hemolink™)

TABLE IV

Comparison of the effects of sham surgery and exchange transfusion with blood, 6% albumin, or SFH solutions on glomerular filtration rate (GFR), renal plasma flow (RPF), renal blood flow (RBF), renal vascular resistance (RVR) and the filtration fraction (FF)

Blood group	GFR (ml/min/300g)	RPF (ml/min/300g)	RBF (ml/min/300g)	RVR (mmHg/ml/min/300g)	FF (%)
Sham group	2.8±0.1	8.2±0.3	15.4±0.6	8.0±0.6	34±1
Cross-linked SFH solutions:	3.2±0.2	9.4±0.5	17.6±0.9	6.4±0.4	33±1
o-raffinose crosslinked SFH #1	2.9±0.1	9.0±0.3	14.0±0.5	9.2±0.4	32±1
o-raffinose crosslinked SFH #2	3.3±0.1	10.0±0.5	15.8±0.8	7.3±0.2	33±1
o-raffinose crosslinked SFH #3	3.2±0.1	10.0±0.4	15.6±0.6	7.6±0.4	32±1
Unmodified SFH	1.8±0.2*†	5.9±0.5*†	9.6±0.9*†	15.9±0.9*†	32±2
Albumin group	3.2±0.1	10.5±0.5*	16.0±0.8	6.7±0.3†	31±1*

The number of experiments in each group are the same as for Table III
*p<0.01 compared to sham operated rats or group exchange tranfused with whole blood
†=p<0.01 compared to group exchanged with *O*-raffinose crosslinked SFH #1

TABLE V

Comparison of the effects of sham surgery and exchange transfusion with blood, 6% albumin, or SFH solutions on on urine flow rate (UV), and on the fractional urinary excretion of sodium and potassium.

	UV (µl/min)	FeNa (%)	FeK (%)
Blood group	5.1±0.2	0.03±0.01	11.7±1.0
Sham group	5.5±1.0	0.02±0.01	14.1±3.3
Cross-linked SFH solutions:			
o-raffinose crosslinked SFH #1	13.8±2.6*	0.14±0.03*	19.1±1.7*
o-raffinose crosslinked SFH#2	9.9±1.7*	0.18±0.07*	22.3±2.2*
o-raffinose crosslinked SFH #3	11.8±2.4*	0.10±0.01*	22.7±2.0*
Unmodified SFH	7.9±1.6	0.23±0.06*	16.3±1.9*
Albumin group	20.8±6.1*	0.17±0.04*	21.4±2.0*

The number of experiments in each group are the same as for Table III
*p<0.05 compared to sham operated group or group exchange transfused with whole blood

TABLE VI

Plasma concentration of hemoglobin Ao and o-raffinose crosslinked SFH solution #1

	Absolute plasma SFH levels (mg/dl)		Reduction in plasma SFH (%)
	10 minutes post-infusion	110 minutes post-infusion	
Ao SFH (n=12)	2.36±0.25	0.36±0.03*	83±3
o-Raffinose cross-linked SFH solution #1 (n=10)	2.97±0.08	2.47±0.09*	17±2†

*p<0.01 compared to 110 minutes
†p<0.01 compared to Ao SFH

TABLE VII

Effect of SFH solutions on platelet NO release

	NO (nM)
GFP	269 ± 20.9
GFP + unmodified (Ao) SFH	4.3 ± 1.8*
GFP + o-raffinose crosslinked SFH # 1	49.7 ± 15.3*†
GFP + o-raffinose crosslinked SFH #2	49.7 ± 14.4*†
GFP + o-raffinose crosslinked SFH #3	59.7 ± 12.5*†

GFP = gel-filtered platelets stimulated with 5μM ADP

(n=3)

*$p<0.05$ compared to GFP; †$p<0.05$ compared to GFP + unmodified SFH

TABLE VIII

Effect of hemoglobin solutions on SNO-GSH induced platelet cGMP production

	pmol cGMP/10^8 platelets
GFP	0.20±0.06
GFP + SNO-GSH	0.35±0.05*
GFP + SNO-GSH + unmodified hemoglobin Ao	0.08±0.01
GFP + SNO-GSH + o-raffinose crosslinked SFH #1	0.21±0.14†
GFP + SNO-GSH + o-raffinose crosslinked SFH #2	0.16±0.06†
GFP + SNO-GSH + o-raffinose crosslinked SFH #3	0.17±0.05†

SNO-GSH = S-nitrosoglutathione

(n=3)

*$p<0.05$ compared with all other samples; †$p<0.05$ compared with GFP SNO-GSH + unmodified (Ao) SFH;

FIGURE LEGENDS

Figure 1: Effect of sham surgery and exchange tranfusion with whole blood, albumin, and SFH solutions on hematocrit.

Blood group (n=16), sham rats (n=5), o-raffinose crosslinked solution rats #1 (n=24), o-raffinose crosslinked solution rats #2 (n=10), o-raffinose crosslinked solution rats #3 (n=10), SFH Ao exchanged rats (n=15) and albumin exchanged rats (n=10).

*=$p<0.01$ vs blood group; †=$p<0.01$ vs o-raffinose crosslinked solutions #1, #2, and #3 as well as the albumin (HSA) solution.

Figure 2: Effect of exchange tranfusion with whole blood (stippled colums), o-raffinose crosslinked solution #1 (cross-hatched columns), and unmodified SFH (black columns) on systemic vascular resistance during each of the four observational periods following the exchange.

*=$p<0.01$ vs blood group; †=$p<0.01$ vs o-raffinose crosslinked solution #1

Figure 3: Effect of SFH solutions on EDRF-induced inhibition of platelet aggregation

Bovine aortic endothelial cells (BAECs) grown on Cytodex beads (ECBs) were incubated without (control) or with 1μM heme in the form of unmodified SFH or o-raffinose crosslinked solution. Platelet aggregation was then induced with 5μM ADP. The percent increase in platelet aggregation above control ECBs (no hemoglobin) induced by each of the SFH solutions is shown.

(n=3)

*$p<0.05$ compared to unmodified SFH

Figure 1 Lieberthal et al

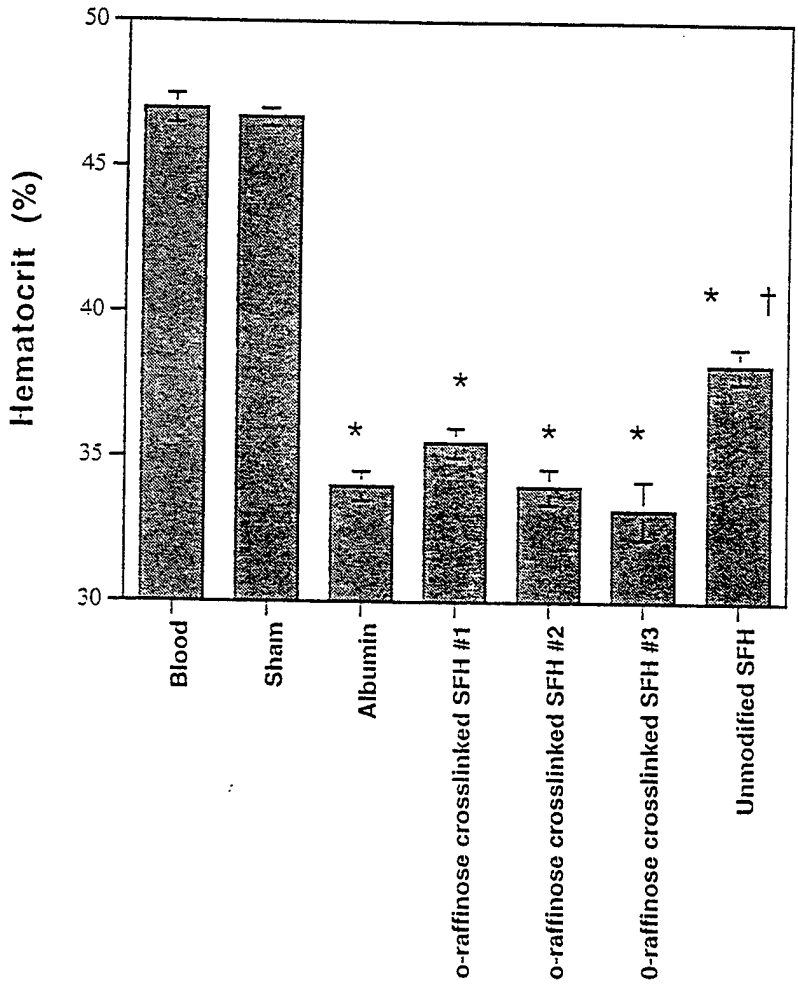

Figure 2 (Lieberthal et al)

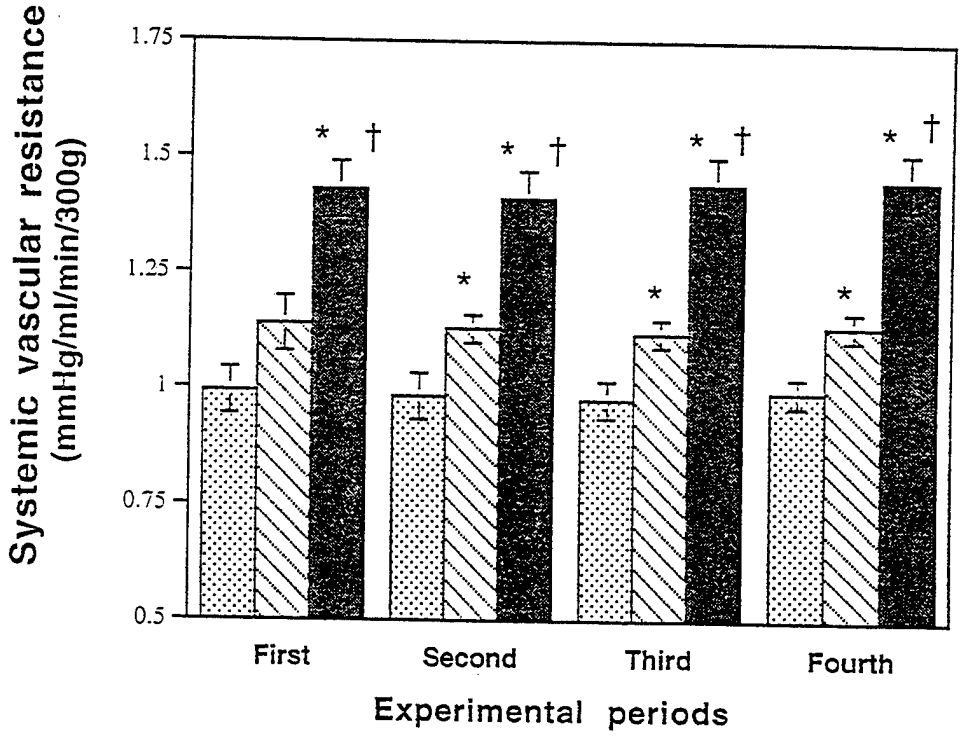

Figure 3 Lieberthal et al